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Abstract

The kainic acid (kainate, KA) receptors belong to the class of ionotropic glutamate (iGlu) receptors in the central nervous system. Five subtypes have been identified, which have been termed $KA_{1,2}$ and $iGlu_{5–7}$. In the search for subtype selective ligands, α-amino-5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA), (4*R*)-methyl Glu (**1a**), and *E*-4-neopentylidene Glu (**2f**) have all previously been reported as selective agonists for the $iGlu_5$ receptor subtype. In this paper, we present the pharmacological evaluation of a five-compound series of (4*R*)-alkyl Glu analogs (**1b**–**e**,**g**) which may be envisaged as conformationally released designs of ATPA and 4-alkylidenes **2a**–**h**. Most notable is the pharmacological profile for (4*R*)-isopentyl Glu (**1g**) which shows a 10-fold increase in binding affinity for the $iGlu_5$ receptor subtype (K_i = 20.5 nM) in comparison with its *E*-4-alkylidene structural isomer **2g**. Furthermore, **1g** displays high selectivity over other KA receptor subtypes ($KA_{1,2}$ and $iGlu_{6,7}$), AMPA-, and NMDA receptors (2050 and > 5000 fold, respectively).

Keywords: Glutamate receptor; Kainate receptor; Subtype selectivity; $iGlu_5$; $iGlu_6$; $iGlu_7$

1. Introduction

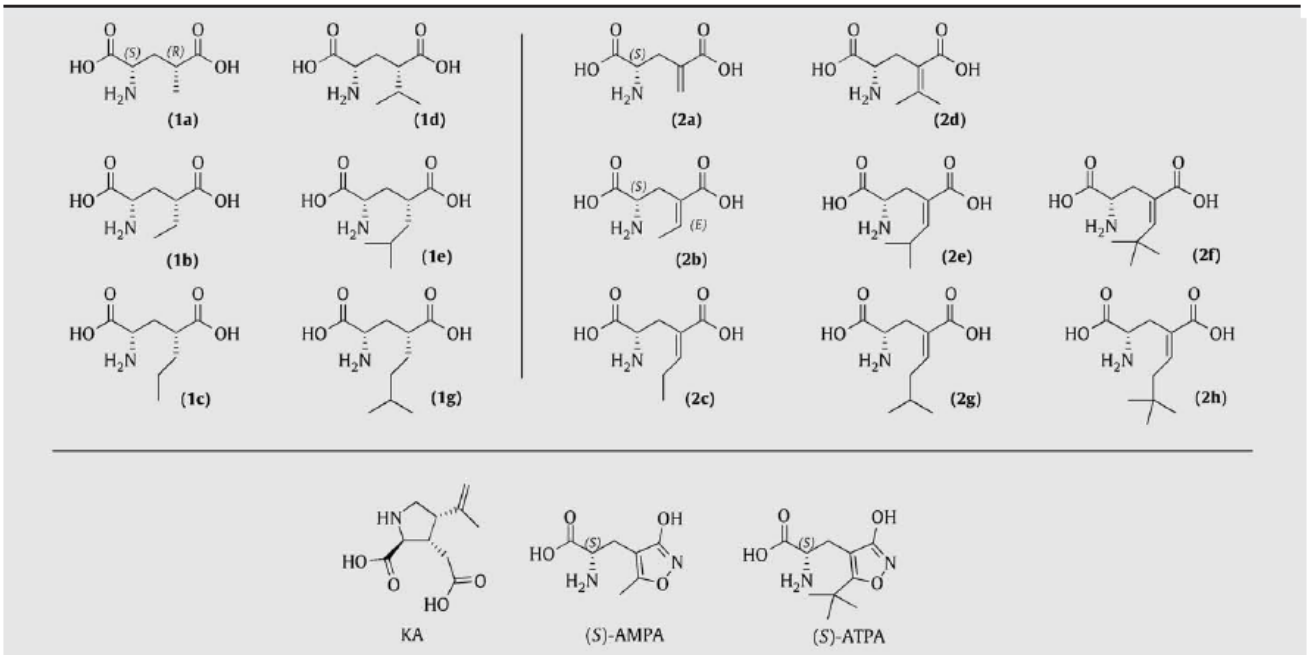
(*S*)-Glutamic acid (glutamate, Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) activating the plethora of ionotropic Glu (iGlu) receptors and metabotropic Glu (mGlu) receptors ([Bräuner-Osborne et al., 2000] and [Meldrum, 2000]). Whereas the iGlu receptors are ion channels and thus mediate a fast excitatory response (Na^+, K^+, Ca^{++} flux), the mGlu receptors are classified as G-protein coupled receptors and generate a slower signal transduction through second messenger systems. On the basis of pharmacological studies, the iGlu receptors are further divided into: 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptors (homo- or heteromeric receptors comprising the subunits $iGlu_{1–4}$), kainic acid (KA) receptors (homo- or heteromeric receptors comprising the subunits $iGlu_{5–7}$ and $KA_{1,2}$) (Wisden and Seeburg, 1993), and the *N*-methyl-d-aspartic acid (NMDA) receptors (heteromeric receptors comprising the subunits NR_1, $NR_{2A–D}$, $NR_{3A–C}$). The mGlu receptors comprise eight homodimeric subtypes, $mGlu_{1–8}$, which are grouped with respect to the second messenger system activated, pharmacology, and molecular biology (group I: $mGlu_{1,5}$ receptors; group II: $mGlu_{2,3}$ receptors; group III: $mGlu_{4,6–8}$ receptors) (Ferraguti and Shigemoto, 2006). Termination of the excitatory signal is controlled by uptake of Glu from the synaptic cleft by the excitatory amino acid transporters (EAATs) (Beart and O'Shea, 2007). To date, five subtypes have been identified of which $EAAT_{1–3}$ are high capacity Glu transporting proteins, $EAAT_4$ functions predominantly as a chloride ion channel and $EAAT_5$ is present exclusively in the retina.

In order to study the function of a single receptor subtype, when found in its intrinsic biological environment, the application of subtype selective ligands (agonists, partial agonists or antagonists) is a useful pharmacological approach. In 1997 the AMPA-analog, α-amino-5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA), was shown to be a highly selective ligand for the $iGlu_5$ receptor subtype. Concurrently (4*R*)-methyl Glu (**1a**) was found to display preference for KA receptors over AMPA, NMDA, and the mGlu receptors ([BraunerOsborne et al., 1997], [Gu et al., 1995], [Sagot et al.,

2008] and [Zhou et al., 1997]), and in 2001 we reported that *E*-4-neopentylidene Glu (**2f**) (Bunch et al., 2001) is a highly selective $iGlu_5$ receptor ligand (Table 1). In this paper we present the pharmacological investigation of a five-compound series of (4*R*)-alkyl Glu analogs (**1b**–**e**,**g**) which may be viewed as conformationally *released* analogs of **2b**–**e**,**g**. Such a distinct – yet confined – change in physical chemical property is interesting as it allows for a detailed investigation of ligand flexibility as opposed to receptor subtype selectivity.

Table 1.

Binding affinities of (4*R*)-alkyl Glu analogs (**1a**–**e**,**g**), *E*-4-alkylidene Glu analogs (**2a**–**h**), AMPA, ATPA, and KA at native iGlu receptors (rat brain synaptosomes) and at cloned rat homomeric $iGlu_{5–7}$ receptor subtypes.



Entry	[^{3}H]AMPA IC_{50} [nM]	[^{3}H]KA[a] IC_{50} [nM]	NMDA K_i [nM]	$iGlu_5$ K_i [nM]	$iGlu_6$ K_i [nM]	$iGlu_7$ K_i [nM]
1a[b]	26,600	32	5900	0.7	17	6
1b	16,000 ± 1000	120 ± 10	28,000 ± 5000	2.08 ± 0.12	75.5 ± 5.7	27.5 ± 9.4
1c	78,000 ± 2000	700 ± 30	>100,000	11.31 ± 0.55	458 ± 29	141.8 ± 0.4
1d	>100,000	1400 ± 100	~75,000 (n = 1)	37.7 ± 0.73	2270 ± 376	383 ± 38
1e	>100,000	5600 ± 200	>100,000	63.5 ± 9.4	4690 ± 434	1596 ± 293
1g	42,000 ± 4000	>100,000	>100,000	20.5 ± 3.4	>100,000	6167 ± 216
2a[b]	150	230	1200	270	450	–
2b[b]	–	–	–	2200	2500	–
2c[b]	–	–	–	61	18,000	–
2d[b]	–	–	–	13,900	>400,000	–
2e[b]	–	–	–	54	83,000	520
2f	2000[b]	>100,000[b]	>100,000[b]	10.4 ± 1.3	>1,000,000	2247 ± 155
2g[b]	–	–	–	224	634,000	–
2h	6900[b]	>100,000[b]	>100,000[b]	122 ± 20	>1,000,000	27,270 ± 3690
AMPA	40[b]	>100,000[b]	>100,000[b]	2000[b]	>100,000[b]	42,700 ± 12,500
ATPA	1800[b]	23,000[b]	>100,000[b]	4[b]	>100,000[b]	2530 ± 250
KA[b]	4000	7	>100,000	76	13	33

[a] $KA_{1,2}$ are the predominant KA receptor subtypes expressed in native rat synaptosomes.

[b] Data taken from original papers: **1a**, ([BraunerOsborne et al., 1997], [Gu et al., 1995], [Sagot et al., 2008] and [Zhou et al., 1997]); **2a**, ([Baker et al., 2000] and [BraunerOsborne et al., 1997]); **2b**–**e**,**g**, (Baker et al., 2000); **2f**,**h**, (Bunch et al., 2001); AMPA, ([Clarke et al., 1997] and [Vogensen et al., 2000]); ATPA, ([Clarke et al., 1997] and [Stensbol et al., 1999]); KA, ([Baker et al., 2000], [Conti et al., 1999] and [Sagot et al., 2008]).

2. Materials and methods

2.1. Binding affinities at native and homomeric ionotropic Glu receptors

Binding affinities for **1b**–**e**,**g** at native AMPA, KA, NMDA receptors (rat synaptosomes) were determined according to the previously published experimental procedure (Hermit et al., 2004) using radioligands [^{3}H]AMPA, [^{3}H]KA (representing predominantly subtypes $KA_{1,2}$), and [^{3}H]CGP39653, respectively. Determination of binding affinities for **1b**–**e**,**g** and **2f**,**h** at cloned rat homomeric receptor subtypes $iGlu_{5–7}$ were carried out following the procedure described earlier, using [^{3}H]-SYM2081 as the radioligand (Sagot et al., 2008).

2.2. Molecular modelling

The modeling study was performed using the software package MOE (Molecular Operating Environment, v2004.03, Chemical Computing Group, 2004) using the build-in mmff94x forcefield and the GB/SA continuum solvent model. Compound **1g** was submitted to a stochastic conformational search and with respect to its global minimum returned (ΔG in kcal/mol), conformations above + 7 kcal/mol were discarded. The γ-carboxylate group was protonated prior to execution of the conformational search, as this gave a larger and thus more reliable number of output conformations. Superimpositions of ligands, were carried out using the built-in function in MOE, by fitting the ammonium group and the two carboxylate groups. The conformation of ATPA was adapted from X-ray structure when bound in the $iGlu_2$ subunit (PDB: 1 nnk).

3. Results

3.1. Binding affinities at native Glu receptor subtypes

In binding assays at native AMPA, KA and NMDA receptors, **1b** shows preference for the KA receptors with decreasing affinity as the 4-substituent is enlarged in size and bulk (**1a**–**e**,**g**). Eventually, compound **1g** displays very low affinity for any of the three iGlu receptor subgroups (42, > 100, > 100 μM, respectively, Table 1).

3.2. Binding affinities at cloned homomeric Glu receptor subtypes

At cloned homomeric $iGlu_{5-7}$ receptor subtypes, Glu analog **1b** displayed a 35- and 10-fold preference for $iGlu_5$ over subtypes $iGlu_{6,7}$, respectively. As the 4*R*-alkyl substituent is increased in length and bulkiness, this trend is strengthened (Table 1 and Fig. 1), and eventually Glu analog **1g** is highly selective for $iGlu_5$ receptor subtype (K_i = 20.5 nM), displaying more than 5000-fold and 300-fold selectivity over $iGlu_{6,7}$ receptor subtype, respectively (Table 2).

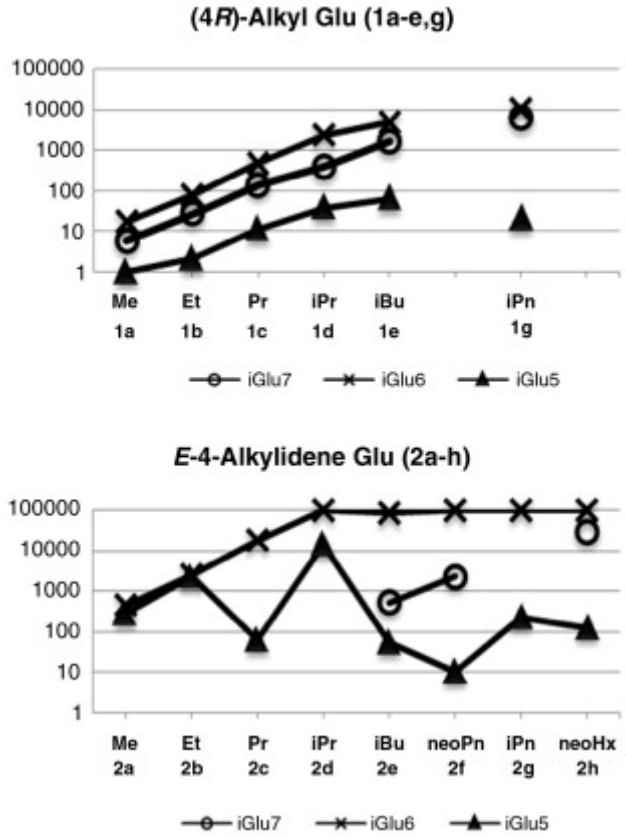


Fig. 1. Binding affinities (pK_i [nM]) of (4*R*)-alkyl Glu analogs **1a**–**e**,**g** (top) and *E*-4-alkylidene Glu analogs **2a**–**h** (bottom) at cloned homomeric $iGlu_{5-7}$ receptor subtypes.

Table 2.

Receptor subtype selectivity calculated and normalized from data presented in Table 1 ($iGlu_5$ receptor subtype set to 1).

	AMPA	**$KA_{1,2}$**[a]	**NMDA**	**$iGlu_5$**	**$iGlu_6$**	**$iGlu_7$**
1g	2050	> 5000	> 5000	1	> 5000	300
2f	80	> 4200	> 4200	1	> 4200	90
2g	–	–	–	1	2830	–
ATPA	450	5750	> 25,000	1	> 25,000	2500

[a] Predominant KA receptor subtypes expressed in native rat synaptosomes.

4. Discussion

Despite the fact that Glu is a highly flexible molecule (Fig. 2), it has been shown in several X-ray crystallographic and medicinal chemistry studies, that Glu agonizes iGlu receptors in a well-defined conformation termed the *folded* conformation ([Bunch and Krogsgaard-Larsen, 2009], [Bunch et al., 2003] and [Hogner et al., 2002]). On the other hand, when activating the mGlu receptors, Glu adopts an *extended* conformation ([Hayashi et al., 1992] and [Kozikowski et al., 1998]).

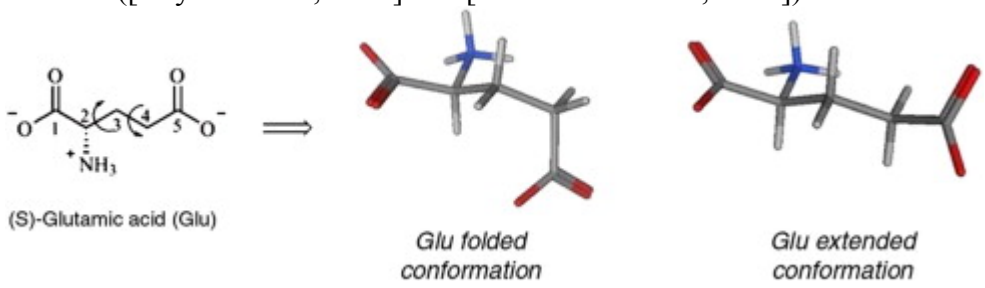


Fig. 2. Rotation of the C(2)–C(3) and C(3)–C(4) bonds allows Glu to adapt nine different staggered conformations. Of these, the two low-energy conformations are termed the Glu *folded* and *extended* conformations. These two Glu conformations are also observed when Glu is crystallized with iGlu receptor subunits (e.g. the $iGlu_5$ subunit: PDB code: 1TXF) and $mGlu_1$ subunit (PDB code: 1EWK).

Recently we reported the synthesis and pharmacological evaluation of a series of (4*R*)-alkyl Glu analogs at $EAAT_{1–3}$ (Alaux et al., 2005). In extension from earlier findings, we showed that introduction of a large variety of longer and more bulky (4*R*)-alkyl substituents also endorses the *folded* conformation as the global low-energy conformation of Glu. In comparison, this low-energy conformational preference is also preferred for *E*-4-alkylidene Glu analogs **2a**–**h**, AMPA, and ATPA (Fig. 3). Furthermore, a general feature for all of these Glu analogs, is the observed increase in preference for the $iGlu_5$ receptor subtype, as the alkyl group is extended in length and bulk (**1a** → **1g**, **2a** → **2f** and AMPA → ATPA; Table 1). This observation may be explained by comparing the size of the iGlu receptor binding pockets: For subunit $iGlu_5$ it has been estimated to be approximately 20% larger as compared to subunit $iGlu_6$ and approximately 50% larger, when compared to subunit $iGlu_2$ (305 Å, 255 Å, and 218 Å, respectively) (Mayer, 2005).

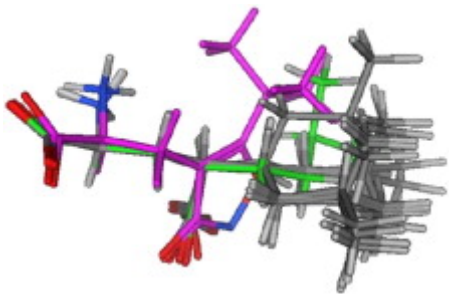


Fig. 3. Superimposition of low-energy conformations of **1g** ($\Delta\Delta G$ = 0 to + 1 kcal/mol) (type code/green) and ATPA (purple). The conformation of **1g** shown in green resembles the conformation of ATPA best.

(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(4*R*)-Alkyl Glu analogs **1a**–**e**,**g** are conformationally released structural analogs of 4-alkylidenes **2a**–**h**. This confined modification of physical chemical property may allow for a detailed investigation of ligand flexibility opposed to receptor subtype selectivity. Comparing the binding affinity profiles of series **1** and **2** at $iGlu_{5-7}$ receptor subtypes reveals that both series display increasing selectivity as well as binding affinity for $iGlu_5$, as the 4-substituents is extended in length and bulk. However, from this resemblance two divergences are noted: Firstly, **1g** vs. **2g** displays a 10-fold difference in binding affinity at the $iGlu_5$ receptor subtype (K_i = 20.5 and 224 nM, respectively). This finding is intriguing and raises the question as to what origin this observation has. One explanation could be that the more flexible ligand, **1g**, may allow for a more tight domain closure, favoring both ligand–receptor and receptor–receptor interactions. A different explanation could be enhanced hydrophobic interactions with the receptor protein. Secondly, **2d** stands out from the trend, displaying significantly lower binding affinity to the $iGlu_5$ receptor subtype as compared to **2c** and **2e**, as well as **1c**–**e**. However, this is explained on the basis that **2d** is structurally quite distinct from preceding analogs **2a**–**c** and subsequent analogs **2e**–**f**, as it has a methyl group in the *Z*-1′ position as opposed to hydrogen.

In conclusion, Glu analogs **1b**–**e**,**g** are a conformationally released structural design of **2a**–**h** and ATPA. Most notably, **1g** displays high affinity for the KA receptor subtype $iGlu_5$ with a high degree of selectivity over receptor subtypes $iGlu_{6,7}$ (fold ratio: > 5000, 300, respectively), KA subtypes KA_1/KA_2 (fold ratio: > 5000, determined by KA binding at native rat synaptosomes), AMPA receptors (2050 fold, determined by AMPA binding at native rat synaptosomes), and NMDA receptors (> 5000 fold, determined by CGP39653 binding at native rat synaptosomes), making **1g** a valuable pharmacological tool. Furthermore, **1g** displays an intriguing 10-fold higher binding affinity at $iGlu_5$ receptor subtype than its corresponding *E*-4-alkylidene isomer **2g**. This observation encourages an expansion of the (4*R*)-alkyl Glu series by the synthesis of corresponding saturated analogs of **2f**,**h**, and a following investigation whether such difference in binding affinity at $iGlu_5$ receptor subtype is a generally observable fact and ultimately if it translates into functional differences.

Acknowledgements

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